DEFECTIVE ADENOVIRUSES AND CORRESPONDING COMPLEMENTATION LINES

The invention relates to new defective adenoviral vectors permitting the transfer and expression of genes of interest to a host eukaryotic cell or organism, as well as to new complementation lines complementing *in trans* the essential viral functions which have been deleted from the genome of these recombinant adenoviruses. The invention is of very special interest for prospects of gene therapy, in particular in man.

Adenoviruses are DNA viruses which display a broad host range. They have been demonstrated in many animal species and many cell types. There are many serotypes which differ in particular in respect of their genome sequence. Most human adenoviruses are only marginally pathogenic and generally produce only benign symptoms.

The adenovirus enters the permissive host cell via a specific receptor, and it is then internalized and passes into endosomes. Their acidification contributes to a change in conformation of the virus and to its emergence into the cytoplasm. The viral DNA associated with certain viral proteins needed for the first steps of the replicative cycle then enters the nucleus of the infected cells, where its transcription is initiated by cellular enzymes. Replication of the adenoviral DNA takes place in the nucleus of the infected cells and does not require cell replication. Assembly of the new virions also takes place in the nucleus. In a first stage, the viral proteins assemble so as to form empty capsids of icosahedral structure, in which the adenoviral DNA is then encapsidated. The viral particles or virions are released from the infected cells and are capable of infecting other permissive cells.

The infectious cycle of the adenovirus takes place in 2 steps:

- the early phase which precedes initiation of the replication of the adenoviral genome, and which

permits production of the regulatory proteins participating in the replication and transcription of the viral DNA, and

- the late phase which leads to the synthesis of the structural proteins.

In general terms, the adenoviral genome consists of a double-stranded linear DNA molecule approximately 36 kb in length which contains the sequences coding for more than 30 proteins. At each of its ends, a short inverted sequence of 100 to 150 nucleotides, depending on the serotypes, designated ITR (inverted terminal repeat), is present. ITRs are involved in the replication of the adenoviral genome. The encapsidation region of approximately 300 nucleotides is located at the 5' end of the genome immediately after the 5' ITR.

The early genes are distributed in 4 regions which are dispersed in the adenoviral genome, designated E1 to E4 (E denoting "early"). The early regions comprise at least six transcription units which possess their own promoters. The expression of the early genes is itself regulated, some genes being expressed before others. Three regions, E1, E2 and E4, respectively, are essential to the viral replication. Thus, if an adenovirus is defective for one of these functions, that is to say if it cannot produce at least one protein encoded by one of these regions, this protein will have to be supplied to it *in trans*.

The E1 early region is located at the 5' end of the adenoviral genome, and contains 2 viral transcription units, E1A and E1B, respectively. This region codes for proteins which participate very early in the viral cycle and are essential to the expression of almost all the other genes of the adenovirus. In particular, the E1A transcription unit codes for a protein which transactivates the transcription of the other viral genes, inducing transcription from the promoters of the E1B, E2A, E2B and E4 regions.

The products of the E2 region, which also comprises two transcription units E2A and E2B, are directly

involved in the replication of the viral DNA. This region governs, in particular, the synthesis of a 72 kDa protein which displays a strong affinity for single-stranded DNA, and of a DNA polymerase.

The E3 region is not essential to the replication of the virus. It codes for at least six proteins which appear to be responsible for inhibition of the host's immune response with respect to an adenovirus infection. In particular, the gp19kDa glycoprotein appears to prevent the CTL response which is responsible for the cytolysis of infected cells by the host's cytotoxic T cells.

The E4 region is located at the 3' end of the adenoviral genome. It codes for many polypeptides which are involved in the expression of the late genes, the stability of late messengers ~~[sic]~~ (mRNAs), the transition from the early phase to the late phase and also the inhibition of cellular protein synthesis.

Once replication of the viral DNA has been initiated, transcription of the late genes begins. These occupy the majority of the adenoviral genome and partially overlap the transcription units of the early genes. However, they are transcribed from different promoters and according to an alternative mode of splicing, so that the same sequences are used for different purposes. Most of the late genes are transcribed from the major late promoter (MLP). This promoter permits the synthesis of a long primary transcript, which is then matured in the form of about twenty messenger RNAs (mRNAs) from which the capsid proteins of the virion are produced. The gene coding for structural protein IX of which the capsid is composed is located at the 5' end of the adenoviral genome and overlaps the E1B region at its 3' end. The protein IX transcription unit utilizes the same transcription termination signal as the E1B transcription unit.

A number of adenoviruses are now well characterized genetically and biochemically. This is the case with human adenovirus type 5 (Ad5), the sequence of which is

disclosed in the ~~Genebank~~ Gen Bank data bank under reference M73260 (SEQ ID NO:43). It has been possible to localize the different genes precisely on the adenoviral genome, which comprises, from 5′ to 3′, the 103-bp 5′ ITR followed by the approximately 300-bp encapsidation region (Hearing et al., 1987, J. Virol., *61*, 2555-2558), then the early and late regions whose location is shown diagrammatically in Figure 1, and lastly the 3′ ITR.

It emerges from the foregoing that adenoviruses possess advantageous features which make them vectors of choice for the transfer of genes of interest. Many recombinant adenoviruses are described in the literature (Rosenfeld et al., 1991, Science, *252*, 431-434; Rosenfeld et al., 1992, Cell, *68*, 143-155). Generally speaking, they are derived from Ad5, and are defective for the E1 function so as to avoid their dissemination in the environment and the host organism. In addition, the non-essential E3 region can also be deleted. Exogenous sequences are integrated in place of the E1 or E3 region.

Thus, these defective adenoviruses can be propagated only in a cell line complementing *in trans* the E1 function which is essential to viral replication. At present, the only complementation line which is usable is the embryonic kidney line 293 (Graham et al., 1977, J. Gen. Virol., *36*, 59-72), which results from the integration in its chromosomes of a fragment of the Ad5 genome comprising, in particular, the 5′ end of the viral genome; so that line 293 complements adenoviruses which are defective for the E1 function. 293 cells contain sequences which are also found in the defective recombinant adenovirus, such as the 5′ ITR, the encapsidation region and the portion at the 3′ end of the E1B region containing sequences coding for the early proteins.

The feasibility of gene transfer using adenoviruses is now established. However, the question of their safety has not yet been settled. In effect, they are capable of transforming some cell lines in culture, which reflects the potentially oncogenic power of some of the expression products of the adenoviral genome,

essentially of the E1 and probably E4 region, at least for some serotypes. Furthermore, the probability of genetic recombination between a defective adenovirus of the prior art, in particular a recombinant adenovirus, and either a natural or wild-type adenovirus (originating from an accidental contamination or from an opportunistic infection of a host organism) or an adenoviral genome fragment integrated in the complementation line 293, is not insignificant. In effect, one recombination event is enough to restore the E1 function and generate a non-defective recombinant adenovirus capable of being disseminated in the environment. It is also possible to envisage the situation where a wild-type natural adenovirus coinfecting the same cell as a defective adenovirus might complement the latter for the E1 function, causing a codissemination of the two viruses. Lastly, some types of eukaryotic cells produce proteins displaying an E1A-like activity, which are also capable of partially complementing the defective adenoviruses which infect them.

It is hence desirable to have at one's disposal efficacious adenoviral vectors affording a minimum of risk, with a view to their use in gene therapy for correcting *in vivo* serious genetic defects and treating certain disorders for which no effective therapeutic approaches are available. The success of gene therapy applied to man is dependent upon their being obtained.

Furthermore, doubts exist regarding the obtaining of line 293. These doubts can be liable to undermine the acceptability of products intended for human use which are derived therefrom. It would be useful to have at one's disposal complementation lines whose origin and history are precisely known, in order to produce recombinant adenovirus particles intended for human use.

There have now been found (1) new defective adenoviral vectors from which certain specific regions of the adenoviral genome have been deleted, and which are better suited to the transfer of an exogenous nucleotide sequence *in vivo*, and (2) new, characterized

complementation lines which are acceptable from a pharmaceutical standpoint and which hence afford all the safety features required for the production of products intended for human use.

The value of these new vectors is that they display an increased cloning capacity permitting the insertion of one or more large genes of interest, and afford maximal safety of use. These deleterious mutations render these adenoviruses incapable of autonomous replication and of cell transformation without impairing their capacity to transfer and express a gene of interest.

Thus, the subject of the present invention is an adenoviral vector which is defective for replication, capable of being encapsidated in a complementation cell, which is derived from the genome of an adenovirus comprising, from 5' to 3', a 5' ITR, an encapsidation region, an E1A region, an E1B region, an E2 region, an E3 region, an E4 region and a 3' ITR, by deletion of:

(i) all or part of the E1A region and the whole of the portion of the E1B region coding for the early proteins; or

(ii) all or part of the E1A region and all or part of at least one region selected from E2 and E4 regions; or

(iii) all or part of the E1A region and a portion of the encapsidation region.

For the purposes of the present invention, the term "deletion" or "lacking" refers to the elimination of at least one nucleotide in the target region, and the deletion can naturally be continuous or discontinuous. All or part is taken to mean either the whole or only a portion of the region in question. Deletions are preferred which prevent the production of at least one expression product encoded by the said region. Hence they may lie in a coding region or a regulatory region such as the promoter region, and may affect at least one nucleotide so as to destroy the reading frame of a gene or render a promoter region non-functional. The deletions in question may also comprise partial deletions of one or

more genes of the said region or of the whole of the region.

An adenoviral vector according to the invention is defective for replication, but capable of being replicated and encapsidated in a complementation cell which provides it *in trans* with the product(s) for which it is defective so as to generate an adenoviral particle (also termed defective adenovirus) which is incapable of autonomous replication in a host cell but nevertheless infectious, since it has the capacity to deliver the vector to a host cell.

According to a first variant, an adenoviral vector according to the invention is derived from the genome of a natural or wild-type adenovirus by deletion of all or part of the E1A region and the portion of the E1B region comprising the whole of the sequences coding for the early proteins. According to a preferred embodiment, the deletion affects the promoter and the sequences coding for the expression products of the E1B region, that is to say the early proteins, and does not include all or part of the transcription termination signal which overlaps the sequences coding for the late protein IX. As regards an adenoviral vector according to the invention derived from a human adenovirus type 5, said deletion comprises at least the sequences lying between nucleotides 1634 and 3509 of the adenoviral genome, the sequence of which is disclosed in the Genebank data bank under the reference M73260. The object of this deletion is to reduce or eliminate sequences which are common to an adenoviral vector according to the invention and the adenoviral genome fragment integrated in a complementation line, for example line 293. Furthermore, it removes from an adenoviral vector according to the invention sequences whose expression products are potentially oncogenic, at least in conjunction with the expression products of the E1A region.

Moreover, an adenoviral vector according to the invention is derived, in addition, from the genome of a natural or wild-type adenovirus by deletion of all or

part:

- of the E3 region and/or
- of the E2 region and/or
- of the E4 region.

It is self-evident that an adenoviral vector according to the invention can contain one of the three deletions listed above, or two of them in any combination, or alternatively all of the deletions.

According to an especially advantageous embodiment, only a portion of the E3 region, and preferably the portion which does not comprise the sequences coding for the gp19kDa protein, is deleted from an adenoviral vector according to the invention. The presence of the sequence coding for the gp19kDa protein in an adenoviral vector according to the invention will enable the infected cells to elude the host's immunological surveillance; an important criterion when the therapeutic protocol necessitates several repeated administrations. The choice will preferably be made to place the sequences coding for gp19kDa under the control of suitable elements permitting their expression in the host cell, namely the elements needed for transcription of said sequences into mRNA and translation of the latter into protein. These elements comprise, in particular, a promoter. Such promoters are well known to a person skilled in the art, and are inserted upstream of said coding sequence by conventional techniques of genetic engineering. The promoter selected will preferably be a constitutive promoter which cannot be activated by one of the expression products of the E1A region. As examples, there may be mentioned the HMG (hydroxymethylglutarylcoenzyme A reductase) gene promoter, SV40 (simian virus 40) virus early promoter, the RSV (Rous sarcoma virus) LTR (long terminal repeat) or the promoter of a PGK (phosphoglycerate kinase) gene of a higher eukaryote.

Moreover, the portion of the E3 region corresponding to the promoter region can optionally be deleted from an adenoviral vector according to the invention, which promoter region will be replaced by a heterologous

promoter region such as one of those mentioned above.

According to a second variant, an adenoviral vector according to the invention is derived from the genome of a natural or wild-type adenovirus by continuous or discontinuous deletion of all or part of the E1A region and all or part of at least the E2 and/or E4 region. Such a deletion makes it possible to increase the possibilities of cloning genes of interest. Moreover, removing all or part of the E4 region also enables sequences coding for potentially oncogenic products to be reduced or eliminated.

As above, an adenoviral vector according to the invention can, in addition, lack all or part of the E1B and/or E3 regions, and especially according to an embodiment as mentioned above (for instance deletion of the portion of the E1B region comprising the whole of the sequences coding for the early proteins and the portion of the E3 region not coding for the gp19kDa protein).

Lastly, according to a third variant, an adenoviral vector according to the invention is derived from the genome of an adenovirus by deletion of all or part of the E1A region and a portion of the encapsidation region.

A partial deletion of the encapsidation region enables the probability of uncontrolled dissemination of an adenoviral vector according to the invention to be reduced significantly when the latter is in the presence of a wild-type adenovirus. Such a deletion enables its encapsidation functions to be affected in such a way that, even in the case of complementation *in trans* of the defective function of the vector by a wild-type adenovirus, it will not be able to be encapsidated efficiently in comparison to the genome of the competing wild-type adenovirus.

The deletions from the encapsidation region will be chosen on the basis of 2 criteria: a reduced capacity for being encapsidated, but simultaneously a residual efficiency compatible with an industrial production. In other words, the encapsidation function of an adenoviral vector according to the invention is substantially

maintained, though to a lesser degree. The attenuation may be determined by conventional titration techniques, by infecting an appropriate line and evaluating the number of lytic plaques. Such techniques are known to a person skilled in the art. In the context of the invention, the encapsidation efficiency is reduced by a factor of 2 to 50, advantageously 3 to 20 and preferably 5 to 10, relative to a control adenovirus having a wild-type encapsidation region.

Naturally, an attenuated adenoviral vector according to the invention can, in addition, comprise at least one or any combination of the deletions mentioned above.

An adenoviral vector according to the present invention is derived from the genome of a natural or wild-type adenovirus, advantageously a canine, avian or human adenovirus, preferably a human adenovirus type 2, 3, 4, 5 or 7 and, as an absolute preference, a human adenovirus type 5 (Ad5). In this latter case, the deletions of the adenoviral vector according to the invention are indicated by reference to the position of the nucleotides of the Ad5 genome which is specified in the ~~Gene-bank~~ GenBank (SEQ ID NO:43) data bank under the reference M73260.

Most particular preference is given to an adenoviral vector according to the invention derived from the genome of a human adenovirus type 5 by deletion of:

(i) the whole of the portion coding for the early proteins of the E1B region and extending from nucleotide 1634 and ending at nucleotide 4047; and/or

(ii) the E4 region extending from nucleotides 32800 to 35826; and/or

(iii) the portion of the E3 region extending from nucleotides 27871 to 30748; and/or

(iv) the portion of the encapsidation region:

- ranging from nucleotide 270 to nucleotide 346, or
- ranging from nucleotide 184 to nucleotide 273, or

- ranging from nucleotide 287 to nucleotide 358.

Preferably an adenoviral vector according to the invention is derived from the genome of a wild-type or natural adenovirus by deletion of at least 18% of the said genome, of at least 22%, of at least 25%, of at least 30%, of at least 40%, of at least 50%, of at least 60%, of at least 70%, of at least 80%, of at least 90% or alternatively of at least 95%, and in particular of 98.5%.

According to an especially preferred embodiment, an adenoviral vector according to the invention is derived from the genome of an adenovirus by deletion of the whole of the adenoviral genome with the exception of the 5' and 3' ITRs and all or part of the encapsidation region. According to this variant, it comprises only the minimum number of viral sequences so as to limit the risks of recombination and the risks of oncogenicity and to have a maximal cloning capacity. Such a vector will then be referred to as a "minimum" adenoviral vector, in which it will then be possible to insert up to 30 kb of exogenous nucleotide sequence. A preferred adenoviral vector according to the invention is derived from a human adenovirus type 5 by deletion of the portion of the viral genome extending from nucleotides 459 to 35832.

In the context of the present invention, an adenoviral vector according to the invention has as its objective the transfer of an exogenous nucleotide sequence to a host cell and its expression therein. "Exogenous nucleotide sequence" is understood to mean a nucleic acid which comprises coding sequences and regulatory sequences permitting the expression of said coding sequences, and in which the coding sequences are sequences which are not normally present in the genome of an adenovirus. The regulatory sequences can be of any origin. The exogenous nucleotide sequence is introduced into an adenoviral vector according to the invention by standard techniques of genetic engineering, between the encapsidation region and the 3' ITR.

An exogenous nucleotide sequence can consist of one or more gene(s) of interest, and preferably of therapeutic interest. In the context of the present invention, a gene of interest can code either for an antisense RNA, or for an mRNA which will then be translated into a protein of interest. A gene of interest can be of genomic type, of complementary DNA (cDNA) type or of mixed type (minigene, in which at least one intron is deleted). It can code for a mature protein, a precursor of a mature protein, in particular a precursor intended to be secreted and accordingly comprising a signal peptide, a chimeric protein originating from the fusion of sequences of diverse origins, or a mutant of a natural protein displaying improved or modified biological properties. Such a mutant may be obtained by mutation, deletion, substitution and/or addition of one or more nucleotide(s) of the gene coding for the natural protein.

A gene of interest may be placed under the control of elements suitable for its expression in a host cell. "Suitable elements" are understood to mean the set of elements needed for its transcription into RNA (antisense RNA or mRNA) and for the translation of an mRNA into protein. Among the elements needed for transcription, the promoter assumes special importance. It can be a constitutive promoter or a regulable promoter, and can be isolated from any gene of eukaryotic or viral origin, and even adenoviral origin. Alternatively, it can be the natural promoter of the gene of interest in question. Generally speaking, a promoter used in the present invention may be modified so as to contain regulatory sequences. As examples, a gene of interest in use in the present invention is placed under the control of the promoter of the immunoglobulin genes when it is desired to target its transfer to lymphocytic host cells. There may also be mentioned the TK-HSV-1 (herpesvirus, type 1 thymidine kinase) gene promoter or alternatively the adenoviral MLP promoter, in particular of human adenovirus type 2, permitting expression in a large number of cell types.

Among genes of interest which are usable in the context of the present invention, there may be mentioned:

- the genes coding for cytokines such as interferon alpha, interferon gamma, interleukins;
- the genes coding for membrane receptors such as the receptors recognized by pathogenic organisms (viruses, bacteria or parasites), preferably by the HIV virus (human immunodeficiency virus);
- the genes coding for coagulation factors such as factor VIII and factor IX;
- the gene coding for dystrophin;
- the gene coding for insulin;
- the genes coding for proteins participating directly or indirectly in cellular ion channels, such as the CFTR (cystic fibrosis transmembrane conductance regulator) protein;
- the genes coding for antisense RNAs or proteins capable of inhibiting the activity of a protein produced by a pathogenic gene, present in the genome of a pathogenic organism, or by a cellular gene, the expression of which is deregulated, for example an oncogene;
- the genes coding for a protein inhibiting an enzyme activity, such as α_1-antitripsin or a viral protease inhibitor;
- the genes coding for variants of pathogenic proteins which have been mutated so as to impair their biological function, such as, for example, trans-dominant variants of the TAT protein of the HIV virus which are capable of competing with the natural protein for binding to the target sequence, thereby preventing the activation of HIV;
- the genes coding for antigenic epitopes in order to increase the host cell's immunity;
- the genes coding for major histocompatibility complex classes I and II proteins, as well as the genes coding for the proteins which are inducers of these genes;

- the genes coding for cellular enzymes or those produced by pathogenic organisms; and
- suicide genes. The TK-HSV-1 suicide gene may be mentioned more especially. The viral TK enzyme displays markedly greater affinity compared to the cellular TK enzyme for certain nucleoside analogues (such as acyclovir or gancyclovir). It converts them to monophosphated molecules, which can themselves be converted by the cellular enzymes to nucleotide precursors, which are toxic. These nucleotide analogues can be incorporated in DNA molecules undergoing synthesis, hence chiefly in the DNA of cells in a state of replication. This incorporation enables dividing cells such as cancer cells to be destroyed specifically.

This list is not restrictive, and other genes of interest may be used in the context of the present invention.

Moreover, according to another embodiment of the invention, an adenoviral vector according to the invention can, in addition, comprise a non-therapeutic gene coding for a protein which trans-activates non-adenoviral transcription. Naturally, the gene(s) of the E1A region coding for a trans-activating protein, the expression of which would run the risk of rendering the adenovirus non-defective, will be avoided. The gene coding for the *Saccharomyces cerevisiae* Gal4 protein will preferably be chosen. Its expression will enable the vector to be propagated in a complementation line such as the one described below. Such a line is more sophisticated, and enables possible problems of toxicity due to the continuous production of adenoviral complementation proteins to be alleviated. The gene coding for a protein which trans-activates transcription may be placed, if necessary, under the control of elements suitable for its expression; for example those which permit the expression of a gene of interest.

The invention also relates to an adenoviral

particle, as well as to a eukaryotic host cell comprising an adenoviral vector according to the invention. Said cell is advantageously a mammalian cell, and preferably a human cell, and can comprise said vector in integrated form in the genome, or preferably in non-integrated (episome) form.

An adenoviral particle according to the invention may be prepared by passage in any complementation line providing *in trans* the functions for which an adenoviral vector according to the invention is defective, for example line 293 of the prior art. These preparation techniques are known to a person skilled in the art (Graham and Prevec, 1991, Methods in Molecular Biology, vol. 7, 109-128, Ed: E.J. Murey, The Human Press Inc.). Optionally, an adenoviral particle according to the invention may be generated in a complementation line according to the invention such as is described below.

Thus, the present invention also relates to a complementation line containing a complementation element, comprising, in particular, a portion of the E1 region of the genome of an adenovirus with the exception of the 5' ITR; said complementation element being capable of complementing *in trans* a defective adenoviral vector and being integrated in the genome of said complementation line or inserted into an expression vector.

In the context of the present invention, the term "complementation line" refers to a eukaryotic cell capable of providing *in trans* the function(s) for which an adenoviral vector is defective. In other words, it is capable of producing the protein or proteins needed for the replication and encapsidation of said adenoviral vector, early and/or late proteins which it cannot itself produce and which are needed for building a viral particle. Naturally, said portion may be modified by mutation, deletion and/or addition of nucleotides, as long as these modifications do not impair its capacity for complementation. Thus, an adenoviral vector which is defective for the E1 function will have to be propagated in a complementation line for E1 (capable of providing *in*

trans the protein or set of proteins encoded by the E1 region which the vector cannot produce), a vector which is defective for the E1 and E4 functions will be propagated in a complementation line for E1 and E4 (providing the necessary proteins encoded by the E1 and E4 regions), and lastly a vector which is defective for the E1, E2 and E4 functions will be propagated in a complementation line for the three functions. As mentioned in the introduction, the E3 region is nonessential, and does not need to be specifically complemented.

A complementation line according to the invention may be derived either from an immortalized cell line capable of dividing indefinitely, or from a primary line. In accordance with the objectives pursued by the present invention, a complementation line according to the invention is useful for the encapsidation of any defective adenoviral vector, and especially a defective adenoviral vector according to the invention. Thus, when the term "defective adenoviral vector" is used below, it should be understood to refer to any defective vector, of the prior art or of the present invention.

"Complementation element" is understood to mean a nucleic acid comprising at least the portion of the adenoviral genome in use in the context of the present invention. It can be inserted into a vector, for example of the plasmid or viral type, for example a retroviral or adenoviral vector or one derived from a poxvirus. The case where it is integrated in the genome of a complementation line according to the invention will nevertheless be preferred. The methods for introducing a vector or a nucleic acid into a cell line, and possibly of integrating it in the genome of a cell, constitute conventional techniques well known to a person skilled in the art, as do the vectors which are usable for such purposes. The complementation element may be introduced into a complementation line according to the invention, beforehand or concomitantly with a defective adenoviral vector.

According to a specific embodiment, a complementation line according to the invention is intended to

complement *in trans* a defective adenoviral vector for the E1 function. Such a line has the advantage of decreasing the risks of recombination since, contrary to the conventional line 293, it lacks the 5' ITR present in the vectors.

In the context of the present invention, a complementation line according to the invention can comprise all or part of the E1A region of the genome of an adenovirus and:

(i) all or part of at least one region of the adenoviral genome selected from the E1B, E2 and E4 regions, or

(ii) all or part of at least two of the E1B, E2 and E4 regions of said genome, or

(iii) all or part of the E1B, E2 and E4 regions of said genome.

In the context of the invention, said regions may be placed if necessary under the control of suitable elements permitting their expression, but it is preferable to place them under the control of their own promoter, which is inducible by the protein which transactivates transcription encoded by the E1A region.

As a guide, a complementation line according to the variant (ii) comprising the E1A, E1B and E4 regions is intended for the preparation of an adenovirus which is defective for the E1 and E4 regions and from which all or part of the corresponding regions has been deleted.

According to an advantageous embodiment, a complementation line according to the invention comprises, in particular, all or part of the E1A region and the whole of the sequences coding for the early proteins of the E1B region.

Moreover, according to a variant of this embodiment, a complementation line according to the invention can, in addition, lack the promoter region of the E1A region. In this case, the portion of the adenoviral genome coding for the early proteins of said E1A region will be placed under the control of a suitable heterologous promoter which is functional in said

complementation line. It can be isolated from any eukaryotic or viral gene. The use of an adenoviral promoter of an early region will, however, be avoided. The promoter in question can be a constitutive promoter. As examples, the SV40 virus, TK-HSV-1 gene and murine PGK gene promoters may be mentioned.

Alternatively, the promoter selected may be regulable and advantageously inducible by a protein which trans-activates non-adenoviral transcription. It can be a promoter isolated from a naturally inducible gene or any promoter modified by the addition of activating sequences (or UAS, standing for upstream activating sequence) responding to said trans-activating protein. More especially, it is preferable to use a promoter which is inducible by the *Saccharomyces cerevisiae* Gal4 protein, and preferably a hybrid promoter consisting of a so-called "minimum" promoter containing only the transcription initiation sequences (TATA box and start site) of a gene of any kind (for example the TK-HSV-1 gene or Ad2 MLP), upstream of which at least one activating sequence of the *Saccharomyces cerevisiae* Gal10 gene has been inserted (Webster et al., 1988, Cell, 52, 169-178). The latter sequence may be synthesized chemically or isolated from the Gal10 gene according to standard techniques of genetic engineering. Thus, the hybrid promoter will be activated, and will induce the expression of the genes encoded by the E1A region placed under its control, only in the presence of the Gal4 protein. The expression products of the E1A region will then, in their turn, be able to induce the expression of the other E1B, E2 and/or E4 early regions optionally included in a complementation line according to the invention. This particular embodiment of the invention avoids the constitutive production (possibly toxic) of the adenoviral proteins needed for complementation. Thus, induction may be triggered in the presence of a defective adenoviral vector according to the invention expressing the Gal4 protein. However, such a line may also be used to prepare any defective adenoviral vector, on condition, however,

of providing the Gal4 protein *in trans*. The means of providing a protein *in trans* are known to a person skilled in the art.

In general terms, a complementation line comprises a portion of the genome of an adenovirus which is advantageously derived from an animal adenovirus such as a canine or avian adenovirus or, preferably, a human adenovirus, and most especially of type 2 or 5.

A complementation line according to the invention comprises, in particular, the portion of the genome of a human adenovirus type 5 extending:

(i) from nucleotide 100 to nucleotide 5297 of the sequence as disclosed in the ~~Genebank~~ GenBank data bank under the reference M73260 (SEQ ID NO:43), or

(ii) from nucleotide 100 to nucleotide 4034, or

(iii) from nucleotide 505 to nucleotide 4034.

Advantageously, the portion of the genome according to (ii) is inserted upstream of a transcription termination signal, such as, for example, the polyadenylation signal of the SV40 virus (simian virus 40) or of the rabbit β-globin gene. Whereas the portion according to (iii), which comprises neither the promoter sequences of the E1A region nor the transcription termination signal of the E1B region, is placed under the control of a suitable promoter, in particular a promoter which is inducible by the Gal4 protein, and of a transcription termination signal, for example that of the rabbit β-globin gene. ~~[sic]~~ Such a complementation line is considered to be especially safe, since it lacks the majority of the sequences in common with a defective adenovirus.

Moreover, a complementation line according to the invention can contain the portion of the E4 region of a human adenovirus type 5 starting from nucleotide 32800 and ending at nucleotide 35826 of the sequence as disclosed in the ~~Genebank~~ GenBank data bank under the reference M73260 (SEQ ID NO:43).

Moreover, a complementation line according to the invention can contain the whole of the genome of a

natural adenovirus, with the exception of the encapsidation region and the 5' and 3' ITRs, and, as an absolute preference, the portion of the genome of a human adenovirus type 5 starting from nucleotide 505 and ending at nucleotide 35826 of the sequence as disclosed in the ~~Genebank~~ GenBank data bank under the reference M73260. For the purposes of the present invention, this portion is placed under the control of a suitable promoter. A promoter which is inducible by the *Saccharomyces cerevisiae* Gal4 protein will preferably be used. Such a line will enable all of the functions essential to the replication and encapsidation of an adenoviral vector which is defective for the E1, E2 and E4 functions, in particular a minimum adenoviral vector according to the invention, to be complemented *in trans*.

According to a preferred embodiment, a complementation line according to the invention can contain a complementation element comprising, in addition, a gene coding for a selectable marker permitting the detection and isolation of the cells containing it. In the context of the present invention, this can be any gene coding for a selectable marker, such genes being generally known to a person skilled in the art, advantageously a gene for resistance to an antibiotic, and preferably the gene coding for puromycin acetyltransferase (pac gene) conferring resistance to puromycin.

In the context of the present invention, the gene coding for a selectable marker may be placed under the control of suitable elements permitting its expression. These can comprise a constitutive promoter, such as the SV40 virus early promoter. However, a promoter which is inducible by the trans-activating protein encoded by the E1A region will be preferred, especially the E2A adenoviral promoter. Such a combination will induce a selection pressure to maintain the expression of the genes of the E1A region in a complementation line according to the invention. For the purposes of the present invention, the promoter selected may be modified by deletion, mutation, substitution and/or addition of nucleotides.

According to an absolutely preferred embodiment, a complementation line according to the invention is derived from a cell line which is acceptable from a pharmaceutical standpoint. "Cell line which is acceptable from a pharmaceutical standpoint" is understood to mean a cell line which is characterized (whose origin and history are known) and/or which has already been used for the large-scale production of products intended for human use (assembly of batches for advanced clinical trials or of batches intended for sale). Such lines are available from bodies such as the ATCC. In this connection, there may be mentioned the Vero African Green monkey kidney and BHK golden or Syrian hamster kidney lines, the A549 human line derived from a lung carcinoma, and the MRC5 human pulmonary, ~~WI38~~ WI 38 human pulmonary and CHO Chinese hamster ovary lines.

Alternatively a complementation line according to the invention can be derived from primary cells, and in particular from retinal cells taken from a human embryo.

The invention also relates to a method for preparing an adenoviral particle according to the invention, according to which:

- an adenoviral vector according to the invention is introduced into a complementation line capable of complementing *in trans* said vector, so as to obtain a transfected complementation line,
- said complementation line is cultured according to suitable conditions for permitting the production of said adenoviral particle, and
- said particle is recovered in the cell culture.

Naturally, the adenoviral particle may be recovered from the cultured supernatant, but also from the cells according to conventional protocols.

Preferably, a method according to the invention employs a complementation line according to the invention.

The subject of the invention is also the therapeutic or prophylactic use of an adenoviral vector, an adenovirus particle, a eukaryotic host cell or a

complementation line according to the invention.

Lastly, the present invention relates to a pharmaceutical composition comprising as therapeutic or prophylactic agent an adenoviral vector, an adenovirus particle, a eukaryotic cell or a complementation cell according to the invention, in combination with a vehicle which is acceptable from a pharmaceutical standpoint.

The composition according to the invention is intended especially for the preventive or curative treatment of disorders such as:

- genetic disorders such as hemophilia, cystic fibrosis or Duchêne's ~~[sic]~~ and Becker type myopathies,
- cancers such as those induced by oncogenes or viruses,
- retroviral diseases such as AIDS (acquired immunodeficiency syndrome resulting from HIV infection), and
- recurrent viral diseases such as herpesvirus-induced infections.

A pharmaceutical composition according to the invention may be manufactured in a conventional manner. In particular, a therapeutically effective amount of a therapeutic or prophylactic agent is combined with a vehicle such as a diluent. A composition according to the invention may be administered by aerosol or via any conventional route in use in the field of the art, especially via the oral, subcutaneous, intramuscular, intravenous, intraperitoneal, intrapulmonary or intratracheal route. The administration may take place in a single dose or a dose repeated one or more times after a certain time interval. The appropriate administration route and dosage vary in accordance with various parameters, for example with the individual being treated or the disorder to be treated, or alternatively with the gene(s) of interest to be transferred. Generally speaking, a pharmaceutical composition according to the invention comprises a dose of adenovirus according to the invention of between 10^4 and 10^{14}, advantageously 10^5 and

10^{13} and preferably 10^{6} and 10^{11}. A pharmaceutical composition, especially one used for prophylactic purposes, can comprise, in addition, an adjuvant which is acceptable from a pharmaceutical standpoint.

The invention also encompasses a method of treatment, according to which a therapeutically effective amount of an adenoviral vector, an adenoviral particle, a eukaryotic cell or a complementation line according to the invention is administered to a patient requiring such treatment.

The present invention is described more completely by reference to the figures which follow and by means of the examples which follow.

Figure 1 is a diagrammatic representation of the genome of the human adenovirus type 5 (represented in arbitrary units from 0 to 100), indicating the location of the different genes.

Figure 2 is a diagrammatic representation of the vector pTG6546.

Figure 3 is a diagrammatic representation of the vector pTG6581.

Figure 4 is a diagrammatic representation of the vector pTG6303.

Figure 5 is a diagrammatic representation of the vectors pTG1660 and pTG1661.

Figure 6 is a diagrammatic representation of the vectors pTG1653, pTG1654 and pTG1655.

Figure 7 is a diagrammatic representation of the vector pTG5913.

Figure 8 is a diagrammatic representation of the vector pTG8512.

Figure 9 is a diagrammatic representation of the vector pTG8513.

Figure 10 is a diagrammatic representation of the vector pTG8514.

Figure 11 is a diagrammatic representation of the vector pTG8515.

EXAMPLES

The examples which follow illustrate only one embodiment of the present invention.

The constructions described below are carried out according to the general techniques of genetic engineering and molecular cloning detailed in Maniatis et al., (1989, Laboratory Manual, Cold Spring Harbor, Laboratory Press, Cold Spring Harbor, NY). The collective steps of cloning employing bacterial plasmids is carried out by passage in *Escherichia coli (E. coli)* strain 5K or BJ, whereas those employing vectors derived from phage M13 are carried out by passage in *E. coli* NM522. As regards the steps of PCR amplification, the protocol as described in PCR Protocols - A guide to methods and applications (1990, edited by Innis, Gelfand, Sninsky and White, Academic Press Inc.) is applied.

Moreover, cells are transfected according to standard techniques well known to a person skilled in the art. The calcium phosphate technique (Maniatis et al., *supra*) may be mentioned. However, other protocols enabling a nucleic acid to be introduced into a cell may also be employed, such as the DEAE-dextran technique, electroporation, methods based on osmotic shocks, microinjection of a selected cell or methods based on the use of liposomes.

The fragments inserted into the different constructions described below are indicated precisely according to their position in the nucleotide sequence of:

- the Ad5 genome, as disclosed in the ~~Genebank~~ GenBank data bank under the reference M73260 (SEQ ID NO:45),
- the adenovirus type 2 (Ad2) genome, as disclosed in the ~~Genebank~~ GenBank data bank under the reference J01949,
- the SV40 virus genome, as disclosed in the ~~Genebank~~ GenBank data bank under the reference J02400.

EXAMPLE 1: Generation of an "attenuated" adenovirus comprising a deletion of a portion of the encapsidation region

1. Construction of an "attenuated" vector comprising a deletion from nucleotide 184 to nucleotide 273 of the encapsidation region

A vector comprising the following is constructed:

- the 5' ITR of the Ad5 genome (from nucleotide 1 to nucleotide 103),
- the Ad5 encapsidation region lying between nucleotides 104 and 458, in which the portion ranging from nucleotide 184 to nucleotide 273 is deleted and the thymine (T) at position 176 is modified to a cytosine (C) in order to create an *Aat*II restriction site,
- a cassette for the expression of a gene of interest comprising, from 5' to 3', the Ad2 MLP (nucleotides 5779 to 6038), the *Kpn*I-*Xba*I-*Hind*III and *Bam*HI restriction sites, the human cDNA coding for the CFTR protein (the amino acid composition corresponds to the sequence published by Riordan et al., 1989, Science, *245*, 1066-1073; with the exception of a valine in place of the methionine at position 470), the *Pst*I, *Xho*I and *Sal*I sites and lastly the SV40 virus transcription termination signal (nucleotides 2665 to 2538), and
- the fragment of the Ad5 genome extending from nucleotide 3329 to nucleotide 6241.

In a first stage, the *Eco*RI *Sma*I fragment isolated from pMLP11 is cloned between the *Eco*RI and *Eco*RV sites of the vector M13TG131 (Kieny et al., 1983, Gene, *26*, 91-99). This construction originates from pMLP10 (Levrero et al., 1991, Gene, *101*, 195-202), and differs from the parent vector by the introduction of an *Sma*I site at the *Hind*III site. The vector M13TG6501 is obtained. The latter is subjected to a directed mutagenesis in order to delete the sequences lying between nucleotides 184 and 273 of the encapsidation region. The

directed mutagenesis is carried out using a commercial kit (Amersham) according to the supplier's recommendations, and employs the oligonucleotide OTG4174 listed under sequence identifier No. 1 (SEQ ID NO: 1). The mutated vector is designated M13TG6502. The encapsidation region thus deleted is reintroduced in the form of an *Eco*RI-*Bgl*II fragment, the *Bgl*II site being rendered blunt by treatment with Klenow DNA polymerase, into the vector pMLP11 digested with *Eco*RI and *Sma*I.

The vector obtained, pTG6500, is partially digested with *Pst*I, treated with phage T4 DNA polymerase and then digested with *Pvu*I. The *Pvu*I-*Hpa*I fragment isolated from pTG5955 (derived from pMLP11) is inserted into this vector. This fragment contains the SV40 virus transcription termination signal and the portion of the Ad5 genome extending from nucleotide 3329 to nucleotide 6241. The vector pTG6505 thus generated is partially digested with *Sph*I, treated with phage T4 DNA polymerase and religated, the purpose of this being to destroy the *Sph*I site located at the 5' end of the polylinker. This results in pTG6511, into which, after *Bam*HI digestion and treatment with Klenow DNA polymerase, human CFTR cDNA is cloned in the form of a blunt-ended fragment generated by *Xho*I-*Ava*I digestion and treatment with Klenow DNA polymerase. pTG6525 is obtained. For guidance, the CFTR cDNA is isolated from a plasmid of the prior art such as pTG5960 (Dalemans et al., 1991, Nature, *354*, 526-528).

2 *Construction of an "attenuated" vector comprising a deletion from nucleotide 270 to nucleotide 346 of the encapsidation region*

The vector M13TG6501 is subjected to a directed mutagenesis employing the oligonucleotide OTG4173 (SEQ ID NO: 2). The mutated fragment is then reintroduced into pMLP11, as described above, to generate the vector pTG6501. The latter is digested with *Sph*I, and treated with phage T4 DNA polymerase and then with *Pvu*I. pTG6546 (Figure 2) is obtained by cloning the *Pvu*I-*Kpn*I fragment (the *Kpn*I site having been rendered blunt) isolated from

pTG6525 and containing human CFTR cDNA.

3 *Construction of an "attenuated" vector comprising a deletion from nucleotide 287 to nucleotide 358 of the encapsidation region*

The vector M13TG6501 is subjected to a directed mutagenesis in order to delete the sequences lying between nucleotides 287 and 358 of the encapsidation region, and to modify the thymines at positions 275 and 276 to guanines in order to introduce an *Nco*I site. The mutagenesis is carried out using the oligonucleotide OTG4191 (SEQ ID NO: 3) to give M13TG6507. The latter is cleaved with *Bgl*II, treated with Klenow DNA polymerase and then digested with *Eco*RI, and the corresponding mutated fragment is purified and introduced into pMLP11 digested with *Eco*RI and *Sma*I. pTG6504 is generated, from which the *Sph*I (site rendered blunt by treatment with phage T4 DNA polymerase)-*Pvu*I fragment is isolated and inserted between the *Kpn*I site (rendered blunt by treatment with T4 polymerase) and *Pvu*I site of pTG6511. pTG6513 is obtained, which is treated with *Bam*HI and Klenow DNA polymerase before inserting the *Ava*I and *Xho*I fragment of pTG5960 to give pTG6526.

4. *Generation of a defective and attenuated recombinant adenovirus*

Defective recombinant adenoviruses are generated by cotransfection into 293 cells of either pTG6525, pTG6526 or pTG6546 linearized with *Cla*I and Ad-dl324 genomic DNA (Thimmappaya et al., 1982, Cell, *31*, 543-551) also digested with *Cla*I, so as to generate a recombinant virus by homologous recombination. After 8 to 10 days, individual plaques are isolated, amplified in 293 cells and analyzed by restriction mapping. Viral stocks (AdTG6525, AdTG6526 and AdTG6546) are assembled, and their titer is determined according to conventional techniques.

The AdTG6546 virus is placed in a competitive situation by coinfection with Ad-CFTR (Rosenfeld et al.,

1992, Cell, *68*, 143-155), which contains a wild-type encapsidation region. 293 cells are infected with 5 pfu (plaque forming units) of Ad-CFTR and 5 pfu of AdTG6546 per cell. In parallel, total viral DNA is isolated by Hirt's method (Gluzman and Van Doren, 1983, J. Virol., *45*, 91-103), and encapsidated viral DNA is isolated after treating the cells with 0.2% deoxydrolate [sic] and then with 10 µg/ml of deoxyribonuclease (DNase) I to remove DNAs not protected in virions. Whereas the amount of total Ad-CFTR and AdTG6546 DNA is identical, there is approximately 3 times as much encapsidated Ad-CFTR DNA as encapsidated AdTG6546 DNA.

The level of expression of the CFTR protein in the cell extracts of AdTG6546-infected 293 cells is measured. The analysis is performed by Western blotting according to the technique described in Dalemans et al. (1991, Nature, *supra*) employing the monoclonal antibody MATG1031, However, any other antibody which recognizes antigenic epitopes of the CFTR protein may be used. A product with an expected molecular mass of approximately 170 kDa is detected. For guidance, the level of production is roughly equivalent to that obtained in cell extracts infected with unattenuated Ad-CFTR virus.

EXAMPLE 2: Generation of a defective adenovirus from which the E1A region and the whole of the sequences coding for the early proteins of the E1B region have been deleted

1. *Production of a recombinant adenovirus for the expression of the CFTR protein (AdTG6581)*

Such an adenovirus is generated from a plasmid vector pTG6581 comprising, from 5' to 3':

- the Ad5 5' ITR (from nucleotides 1 to 103),
- the Ad5 encapsidation region (from nucleotides 104 to 458),
- an exogenous nucleotide sequence containing an expression cassette which comprises the following elements:

- the Ad2 MLP (nucleotides 5779 to 6038), followed by three tripartite leaders, also of Ad2 (nucleotides 6039-6079; nucleotides 7101-7175; nucleotides 9637-9712); these leaders are included in order to increase the efficiency of translation of the sequences inserted downstream,
- a polylinker comprising, from 5' to 3', the *Xba*I *Hind*III, *Bam*HI, *Eco*RV, *Hpa*I and *Not*I restriction sites which are usable for the cloning of a gene of interest,
- a gene of interest, such as the gene coding for the CFTR protein,
- the transcription termination signal isolated from the SV40 virus (nucleotides 2543 to 2618),
- the portion of the Ad5 adenoviral genome ranging from nucleotides 4047 to 6241.

The fragment of the Ad5 genome extending from nucleotide 4047 to nucleotide 4614 is amplified by PCR from Ad5 genomic DNA. The PCR reaction employs the sense primer OTG5021 (SEQ ID NO: 4) comprising at its 5' end a *Bam*HI site intended to facilitate the subsequent cloning steps, and the antisense primer OTG5157 (SEQ ID NO: 5). The fragment thus generated is treated with Klenow DNA polymerase before being cloned into the *Sma*I site of M13mp18 (Gibco BRL), giving rise to M13TG6517. The sequence of the fragment generated by PCR is verified according to the standard enzymatic method (Sanger et al., 1977, Proc. Natl. Acad. Sci. USA, 74, 5463).

Separately, the *Pvu*I-*Sma*I fragment is isolated from pMLP11. It is cloned between the *Pvu*I and *Kpn*I sites of pTG6511 (Example 1.1), the *Kpn*I site having been rendered blunt by treatment with phage T4 DNA polymerase according to standard methods. The vector pTG6547 is thereby generated.

The latter is digested with the enzymes *Sal*I and *Bst*XI and ligated to two fragments, on the one hand the purified *Bam*HI-*Bst*XI fragment of M13TG6517, and on the other hand the *Xho*I-*Bgl*II fragment of pTG6185. The latter comprises, in particular, the SV40 virus transcription

termination signal flanked by the *Xho*I and *Bgl*II restriction sites. However, any other plasmid containing the same termination sequence and appropriate restriction sites could be used. The vector pTG6555 is obtained, into which an adapter containing two restriction sites generating blunt ends, *Eco*RV and *Hpa*I, is inserted in the unique *Bam*HI site. This adapter originates from the recombination of the oligonucleotides OTG5564 and OTG5565 (SEQ ID NO: 6 and 7). pTG6580 is obtained. Lastly, the *Sac*I-*Pst*I fragment of pTG6525, the ends of which have been rendered blunt and which contains human CFTR cDNA, is cloned into the *Eco*RV site of pTG6580. pTG6581 (Figure 3) is generated.

The corresponding recombinant adenovirus AdTG6581 is generated by cotransfection of pTG6581 and Ad dl324, both cleaved with *Cla*I, into a complementation line for the E1 function, for instance line 293 or a line from Example 6, according to the standard protocol.

2. *Production of a recombinant adenovirus for the expression of IFN-γ*

The vector pTG6303 (Figure 4) is obtained by cloning the *Hpa*I-*Sma*I fragment of M13TG2437 into the *Hpa*I site of pTG6580. The aforementioned fragment originates from the cloning of the gene coding for interferon gamma (IFN-γ), the sequence of which is as specified in Gray et al., (1982, Nature, *295*, 503-508), into a vector M13TG130 (Kieny et al., 1983, *supra*). The recombinant adenovirus AdTG6303 is obtained according to standard techniques, by homologous recombination resulting from the cotransfection of pTG6303 and the Ad dl324, which is linearized with *Cla*I, into a complementation line for the E1 function.

3. *Construction of an adenovirus from which the E1 region has been deleted and in which the E3 region is placed under the control of a constitutive promoter*

The vector pTG1670 is obtained by cloning a PCR

fragment containing the RSV virus (Rous sarcoma virus) 3' LTR (long terminal repeat) between the *Aat*II and *Bam*HI sites of the vector p polyII (Lathe et al., 1987, Gene, 57, 193-201). The PCR reaction employs the vector pRSV/L (De Wet et al., 1987, Mol. Cell. Biol. 7, 725-737) as a template and the primers OTG5892 and OTG5893 (SEQ ID NO: 8 and 9).

Separately, the 5' portion of the E3 region (nucleotides 27588 to 28607) is amplified by PCR from the vector pTG1659 and using the primers OTG5920 and OTG5891 (SEQ ID NO: 10 and 11). The latter vector is constructed in several steps. The *Bam*HI-*Avr*II fragment (nucleotides 21562 to 28752) is obtained from Ad5 genomic DNA, and then cloned between the same sites of pTG7457 to generate pTG1649. The vector pTG7457 is a pUC19 (Gibco BRL) modified in the polylinker so as to contain, in particular, an *Avr*II site. The *Eco*RI (Klenow)-*Avr*II fragment of M13TG1646 (Example 8) is then introduced into pTG1649 cleaved with *Avr*II-*Nde*I (Klenow), giving the vector pTG1651. Lastly, pTG1659 is generated by inserting the purified *Avr*II fragment (nucleotides 28752 to 35463) of Ad5 genomic DNA into pTG1651 linearized with *Avr*II. The PCR fragment is integrated between the *Xba*I and *Bam*HI sites of p poly II, to give pTG1671. An *Eco*RV-*Aat*II fragment obtained from pTG1670 is then inserted into the *Aat*II site of pTG1671, to give pTG1676.

The *Eco*RI fragment of Ad5 corresponding to nucleotides 27331 to 30049 is isolated from a genomic DNA preparation and subcloned into pBluescript-Sk$^+$ (Stratagene) previously cleaved with *Eco*RI. pTG1669 is obtained. The latter is mutated (Amersham kit) by introducing a *Bam*HI site either at position 27867 (mutagenic oligonucleotide OTG6079; SEQ ID NO: 12) or at position 28249 (mutagenic oligonucleotide OTG6080; SEQ ID NO: 13). pTG1672 and pTG1673, respectively, are obtained. The *Bam*HI-*Bsi*WI fragment, containing the RSV 3' LTR followed by the 5' portion of the E3 region, is isolated from the vector pTG1676, and inserted between the *Bam*HI site (position 27331 or 30049) and *Bsi*W site (position 28390)

of the vectors obtained in the preceding step, to generate pTG1977 and pTG1978. The *Eco*RI fragment obtained from each of these two vectors is then integrated in pTG1679, as a replacement for the wild-type *Eco*RI fragment. pTG1679-E3+ is obtained. For guidance, the vector pTG1679 results from the cloning of the *BstE*II-*Kpn*I fragment (site rendered blunt by treatment with T4 polymerase) of pTG6590 (Example 3.1) between the *Bst*EII site and the *Bam*HI site (site rendered blunt by treatment with Klenow polymerase) of pTG6584 (Example 3.1).

An adenovirus particle is generated by homologous recombination, in a complementation line for the E1 function, between the *Aat*II fragment of pTG1679-E3+ and an adenoviral vector such as Ad dl324 or Ad-RSVβ-gal. The latter contains the β-galactosidase gene in place of the E1 region (Stratford-Perricaudet et al., 1992, J. Clin. Invest., *90* 626-630).

EXAMPLE 3: Construction of a recombinant adenoviral vector having improved cloning capacity by partial deletion of the E1 and E3 regions

1. *Construction of pTG6590ΔE3*

The fragment carrying the portion of the Ad5 genome lying between nucleotides 27325 and 27871 is amplified by PCR from an Ad5 genomic DNA preparation and using the primers OTG6064 and OTG6065 (SEQ ID NO: 14 and 15). OTG6065 comprises at its 5' end a *Bsm*I site, which is also present in the E3 region (at position 30750).

The amplified fragment is cloned into the *Sma*I site of M13mp18, to give M13TG6523. The *Eco*RI-*Bsm*I fragment is isolated from the latter and introduced into the vector pTG6590 cleaved with the same enzymes. pTG6590Δ3 is obtained, which contains the 3' portion of the adenoviral genome (from nucleotides 27082 to 35935), from which portion the E3 region lying between nucleotides 27872 and 30740 has been deleted, whereas a smaller portion of the E3 region (position 28592 to 30470) has been deleted from pTG6590. The vector pTG6590 is obtained

in the following way: a fragment extending from nucleotides 35228 to 35935 (containing the 3' ITR) is generated by PCR from an Ad5 genomic preparation and by means of the primers OTG5481 and OTG5482 (SEQ ID NO: 16 and 17). This fragment is then cloned into the *Sma*I site of M13mp18 to give M13TG6519. Separately, the vector pTG6584 is digested with *Xba*I and then religated in order to remove the corresponding fragment of the E3 region. pTG6589 is obtained, which is cleaved with *Bam*HI, treated with Klenow and then digested with *Bst*EII. The purified *Eco*RI (Klenow)-*Bst*EII fragment of M13TG6519 is introduced into the vector thus treated, to generate pTG6590.

For guidance, the vector pTG6584 is a pUC19 vector (Gibco BRL) which contains the Ad5 sequences extending from the unique *Spe*I site (position 27082) to the beginning of the promoter region of the E4 region (position 35826). It is obtained by digesting pTG1659 (Example 2.3) with *Sal*I and *Spe*I, treated with Klenow DNA polymerase followed by religation.

2. *Construction of an adenoviral vector from which the E1 region and the portion of E3 not expressing the gp19kDa protein have been deleted*

The portion of the E3 region of Ad5 coding for gp19kDa (nucleotides 28731 to 29217) is obtained by PCR from an Ad5 genomic DNA preparation and employing the primers OTG5455 and OTG5456 (SEQ ID NO: 18 and 19). The fragment generated is introduced into the *Sma*I site of M13mp18 to give M13TG6520. The *Eco*RI-*Xba*I fragment of the latter is isolated and cloned into the *Aat*II site of pTG1670 (Example 2.3), the sites having been rendered blunt by treatment with Klenow DNA polymerase. The purified *Xba*I fragment of the vector of the preceding step is then inserted into the *Xba*I site of the vector pTG6590ΔE3 (Example 3.1).

3. *Production of adenoviral particles*

The recombinant viral particles are obtained by ligation of the *Spe*I fragments isolated from AdTG6303 or

AdTG6581 genomic DNA and one or other of the vectors of Examples 3.1 and 3.2. The ligation mixture is then transfected into a complementation line for the E1 function.

EXAMPLE 4: Construction of an adenovirus from which the E1 and E4 regions have been deleted

The portions of the adenoviral genome extending from nucleotides 31803 to 32799 and 35827 to 35935 are amplified from an Ad5 genomic DNA preparation and using the primers OTG5728 and OTG5729 (SEQ ID NO: 20 and 21) and OTG5730 and OTG5781 (SEQ ID NO: 22 and 16), respectively. After about ten amplification cycles, the reaction is continued on the basis of an aliquot of the two reaction mixtures, employing the oligonucleotides OTG5728 and OTG5781. The amplified fragment extends from nucleotides 31803 to 35935, with a deletion of the whole of the E4 region (positions 32800 to 35826). After *Eco*RI and *Hind*III digestion, it is cloned between the same sites of M13mp18 to give M13TG6521.

M13TG6521 is digested with *Eco*RI, treated with Klenow DNA polymerase and then cleaved with *Bst*XI. The 0.46-kb fragment containing the 3' ITR is inserted between the *Bam*HI site, rendered blunt by treatment with Klenow DNA polymerase, and the *Bst*XI site of pTG6584 (Example 3.1). pTG6587 is obtained, which is digested with *Xba*I and then religated with itself, to give pTG6588 (deletion of E3).

A synthetic DNA fragment originating from the recombination of the oligonucleotides OTG6060, OTG6061, OTG6062 and OTG6063 (SEQ ID NO: 23 to 26) is introduced into the *Pac*I site of pTG6588. This results in pTG8500, in which the transcription termination signals of the L5 late genes are improved.

An adenoviral particle (AdΔE4), having a genome from which the whole of the E4 region (nucleotides 32800 to 35826) and the *Xba*I fragment of the E3 region (nucleotides 28592 to 30470) have been deleted, is generated by

ligation of the *Spe*I fragments isolated from pTG8500 or pTG6588 and from Ad5. The ligation mixture is transfected into a complementation cell line for the E4 function, for example line W162 (Weinberg and Ketner, 1983, Proc. Natl. Acad. Sci. USA, *80*, 5383-5386). An adenovirus which is defective for the E1 and E4 functions (ΔE1, ΔE4) is obtained by transfection into a complementation line for E1 and E4 (for example the line of Example 8) of the ligation mixture between the Ad dl324 genome and plasmid pTG8500 or pTG6588 linearized with *Spe*I.

Moreover, it is also possible to proceed in the following manner: the *Spe*I-*Sca*I fragment isolated from pTG1659 (Example 2.3) is cloned into the vector pTG6588 cleaved with these same enzymes, to obtain pTG6591. The latter contains the Ad5 sequences from nucleotides 21062 to 35935 but from which, as above, the whole of the E4 region and the *Xba*I fragment of the E3 region have been deleted. The synthetic DNA fragment described above is introduced into the vector pTG6591 digested with *Pac*I, and pTG6597 is generated. The adenoviral particles may be obtained by homologous recombination between Ad dl324 genomic DNA cleaved with *Spe*I and plasmids ~~[sic]~~ pTG6591 or pTG6597 cleaved with *Bam*HI.

EXAMPLE 5: Construction of a "minimum" virus

A so-called "minimum" adenoviral vector is formed by cloning the following elements into a plasmid:

- the Ad5 5' ITR (from nucleotides 1 to 103);
- the Ad5 encapsidation region (from nucleotides 104 to 458);
- an exogenous nucleotide sequence comprising:
  - a first gene of therapeutic interest, preferably placed under the control of its own promoter in order to obtain a regulation of expression which is as close as possible to the natural regulation,
  - a second gene of interest consisting of the TK-HSV-1 gene, and

- optionally, nucleotide sequences of any kind, added for reasons of efficiency of replication or of encapsidation so that the total size of the genome to be encapsidated is between 30 and 36 kb;
- the sequences coding for the *Saccharomyces cerevisiae* Gal4 protein (Laughon and Gesteland, 1984, Mol. Cell. Biol., *4*, 260-267), placed under the control of a promoter which is functional in a higher eukaryotic cell; and

- The Ad5 3' ITR (from nucleotides 35833 to 35935).

Assembly of these different elements is carried out according to standard techniques of molecular biology. The production of infectious virions comprising such a vector is carried out as described above in a complementation line of Example 7.

EXAMPLE 6: Formation of a complementation cell capable of complementing *in trans* the E1 function

1. *Formation of a complementation cell comprising the E1 region from nucleotides 100 to 5297 (pTG6533)*

This cell contains:

- a cassette for the expression of the pac gene, which gene is placed under the control of the SV40 virus early promoter (nucleotides 5171 to 5243) and comprises at the 3' end the SV40 transcription termination signal (nucleotides 2543 to 2618). The pac gene used corresponds to a fragment ranging from nucleotide 252 to nucleotide 905 of the sequence disclosed by Lacalle et al. (1989, Gene, *79*, 375-380) and containing 4 mutations relative to the published sequence (C at position 305 replaced by A; C at position 367 replaced by T; insertion of a G at position 804; deletion of a G at position 820),
- a fragment of the Ad5 genome ranging from nucleotides 100 to 5297. This fragment comprises the E1A and E1B regions, equipped with their own promoter and their transcription termination signal, as well

as a fraction of the E2 region, thus overlapping the sequences coding for protein IX. For guidance, it appears that line 293 is not capable of producing a functional protein IX.

The construction is carried out in several steps detailed below. The vector p polyIII-I* (Lathe et al., 1987, Gene, *57*, 193-201) is subjected to digestion with the enzymes *Acc*I and *Eco*RI. The *Eco*RI-*Cla*I fragment isolated from plasmid pTG6164 is cloned into the vector thus treated. The vector pTG6528 is obtained.

Plasmid pTG6164 originates from pLXSN (Miller D, 1989, Bio/Techniques, 7, 980) and comprises the pac gene placed under the control of the SV40 virus early promoter. Briefly, the *Hind*III-*Kpn*I fragment of pLXSN is introduced into M13TG131 to produce M13TG4194. The *Nhe*I-*Kpn*I fragment of pMPSV H2 K IL2R (Takeda et al., 1988, Growth Factors, *1*, 59-66) is inserted into the latter, digested with *Nhe*I and *Kpn*I, to produce M13TG4196. The latter is digested with *Hind*III-*Kpn*I, and the purified fragment of pLXSN originating from a *Hind*III digestion and a partial *Kpn*I digestion is cloned. pTG5192 is obtained. The latter is digested with *Hind*III and partially with *Nhe*I, and the *Hind*III-*Nhe*I fragment of pBabe Puro (Land et al., 1990, Nucleic Acids Res., *18*, 3587) is introduced, giving rise to pTG6164.

The vector pTG6528 is digested with *Pst*I, and the *Pst*I fragment isolated from pTG6185 (Example 2.1) containing the SV40 transcription termination signal is introduced at this site. pTG6529 is obtained. The latter is subjected to *Eco*RI-*Hpa*I digestion and ligated to two fragments, on the one hand a purified *Bsp*EI-*Bcg*I fragment (positions 826 to 5297) of Ad5 genomic DNA, and on the other hand a fragment generated by PCR at the *Eco*RI and *Bsp*EI ends, to give pTG6531. The PCR fragment is generated by gene amplification from Ad5 genomic DNA and the primers OTG4564 and OTG4565 (listed under SEQ ID NO: 27 and 28). The amplified fragment is digested with the enzymes *Eco*RI and *Bsp*EI, and ligated as described in the preceding paragraph.

The vector pTG6531 comprises the 2 transcription units (that of the E1 region and that of the pac gene) in the same orientation. To avoid interference in respect of transcription, they are placed in a head-to-tail (reverse with respect to one another) orientation by treating pTG6531 with *Bam*HI and religating. The vector pTG6533 corresponds to a clone displaying the reverse orientation of the two units.

The vector pTG6533 is transfected into a mammalian cell line, for example the Vero (ATCC, CCL81) or A549 (ATCC, CCL185) line by the calcium phosphate technique. The transfected cells are cultured according to the supplier's recommendations and are placed for 24 hours after transfection in selective medium containing puromycin (concentration 6 μg/ml). Resistant clones are selected, on which the expression of the genes of the E1 region is evaluated in order to determine the most productive clone, which may be used as a complementation line for the preparation of an adenovirus which is defective for the E1 function, such as that detailed in Example 2.

The expression of the sequences coding for the early proteins of the E1 region is analyzed by Northern blotting using suitable probes labeled with the isotope ^{32}P. Production of the proteins encoded by the E1A region is detected by immunoprecipitation after labeling the cells with the isotope ^{35}S and using a commercial antibody (Oncogene Science Inc., reference DP11).

It is also possible to verify the ability of the expression products of the E1A region to activate the promoter of the E1B region (by Northern blot analysis of the E1B mRNAs), or to activate the promoter of the E2 region (by assaying the enzymatic activity after transient transfection of a "reporter" plasmid comprising the CAT (chloramphenicol acetyltransferase) gene placed under the control of the E2 promoter).

Lastly, it is possible to infect these cells with Ad-RSV-βgal (Stratford-Perricaudet et al., 1992, *supra*) and to titrate the virus by the agar technique as soon as

a cytopathic effect is observed. In general, the procedure is as follows: the cells are infected at a moi (multiplicity of infection) of 10. Approximately 48 hours after infection, when the cytopathic effect is visible, the cells are lysed and the β-galactosidase activity is assayed according to the conventional protocol (see, for example, Maniatis et al., 1989, *supra*). Positive clones are reinfected at a lower moi. 48 hours after infection, the supernatant and the cells are harvested according to standard techniques. The viral titer is determined by the agar overlayer method using 293 cells. The ratio of the titer obtained to the initial titer constitutes the amplification factor.

2. Construction of a complementation line comprising the E1 region from nucleotides 505 to 4034 (pTG6557, pTG6558, pTG6559, pTG6564 and pTG6565

The vectors pTG6557, pTG6558 and pTG6559 comprise:

(i) a cassette for the expression of the pac gene (nucleotides 252 to 905 as before) under the control of:

- the Ad2 E2A promoter (nucleotides 27341 to 27030) (in pTG6558),
- the Ad2 E2A promoter from which the sequences lying between nucleotides 27163 and 27182 have been deleted (for pTG6557). Such a mutation enables the baseline level of the E2A promoter to be decreased without affecting the inducibility by the trans-activating protein encoded by E1A, or
- the SV40 early promoter for pTG6559.

In all three cases, it also contains at the 3' end the SV40 virus transcription termination signal (nucleotides 2543 to 2618); and

(ii) an expression cassette containing the portion of the Ad5 E1 region ranging from nucleotides 505 to 4034. This portion of the adenoviral genome contains the whole of the sequences coding for the early proteins

of the E1A region, the transcription termination signal of the E1A unit, the E1B promoter (inducible by the trans-activating protein encoded by E1A) and the whole of the coding sequences of the E1B region. It also includes the sequences coding for protein IX, which overlap the E1B region. However, it lacks the promoter of the E1A region and the transcription termination signal of the E1B and IX transcription units. In order to permit the expression of the sequences of the E1 region, the murine PGK gene promoter is introduced at the 5' end of the adeno-viral fragment, and the transcription termination signal of the rabbit β-globin gene (nucleotides 1542 to 2064 of the sequence disclosed in the ~~Genebank~~ GenBank data bank under the reference K03256) is introduced at the 3' end.

Optionally, nucleotide sequences of any kind, for example isolated from pBR322 (Bolivar et al., 1977, Gene, *2*, 95-113), may also be introduced between the cassettes for the expression of the pac gene and of the E1 region, in order to avoid possible interference with transcription.

The construction of these vectors is performed in several steps reported below.

First, the portion of the Ad5 genome ranging from nucleotide 505 to nucleotide 826 is amplified by PCR from a genomic preparation and using the primers OTG5013, which comprises at the 5' end a *Pst*I site which is useful for the subsequent cloning steps (SEQ ID NO: 29), and OTG4565 overlapping the *Bsp*EI site (SEQ ID NO: 28). The fragment generated by PCR is treated with Klenow DNA polymerase and then introduced into the *Sma*I site of M13mp18, giving rise to M13TG6512. The sequence of the PCR fragment is verified.

The vector pTG6533 (Example 6.1) is digested with the enzymes *Eco*RI and *Bsp*EI. The vector thus treated is ligated with, on the one hand the *Pst*I-*Bsp*EI fragment isolated from M13TG6512, and on the other hand the *Eco*RI-*Pst*I fragment isolated from pKJ-1. The latter

fragment comprises the portion of the murine PGK gene promoter lying between nucleotides -524 and -19, the sequence of which is reported in Adra et al. (1987, Gene, *60*, 65-74). This step gives rise to pTG6552, and enables the murine PGK gene promoter to be inserted upstream of the E1 region of Ad5 beginning at nucleotide 505.

Separately, the *Xho*I-*Bam*HI fragment, of which the end generated by *Xho*I is rendered blunt following treatment with Klenow DNA polymerase, is purified from pBCMG Neo (Karasuyama et al., 1989, J. Exp. Med., *169*, 13-25). This fragment, which comprises the transcription termination signal of the rabbit β-globin gene, is introduced between the *Sma*I and *Bam*HI sites of the vector p polyII-Sfi/Not-14* (Lathe et al., 1987, Gene, 57, 193-201). The vector pTG6551 which results is, for its part, digested with the enzymes *Sph*I and *Eco*RV in order to insert into it a fragment of Ad5 genome ranging from nucleotide 3665 to nucleotide 4034. This fragment is generated by PCR according to the standard protocol. The procedure used employs an Ad5 genomic DNA preparation as template, and the primers OTG5015 which overlaps the internal *Sph*I site at position 3665 (SEQ ID NO: 30) and OTG5014 comprising at the 5' end a *Bgl*II site (SEQ ID NO: 31).

The PCR fragment is treated with Klenow DNA polymerase before being cloned into the *Sma*I site of M13mp18, generating M13TG6516. After verification of its sequence, the PCR fragment is abstracted by *Bgl*II digestion, treatment with Klenow DNA polymerase and *Sph*I digestion. It is inserted between the *Sph*I and *Eco*RV sites of pTG6551. This results in pTG6554.

Separately, the vector pTG6529 (Example 6.1) is subjected to digestion with the enzymes *Hpa*I and *Hind*III. The 2.9-kb fragment containing the pac gene followed by the SV40 virus transcription termination signal is purified. This fragment is ligated to the *Sma*I-*Hind*III fragment isolated from pE2 Lac (Boeuf et al., 1990, Oncogene, *5*, 691-699) which carries the Ad2 E2A promoter. The vector pTG6556 is obtained. Alternatively, it may be

ligated to the *Sma*I-*Hind*III fragment isolated from pE2 Lac D9170 (Zajchowski et al., 1985, EMBO J., 4, 1293-1300), which carries the mutated E2A promoter of Ad2. In this case, pTG6550 is obtained.

pTG6556 is digested with the enzymes *Eco*RI and *Bam*HI . The *Eco*RI-*Sac*II fragment isolated from pTG6552 and the *Sac*II-*Bam*HI fragment isolated from pTG6554 are inserted between these sites. The vector pTG6558 is obtained. The same step carried out on pTG6550 and pTG1643 (Example 7.1) generates pTG6557 and pTG6559, respectively.

pTG6557 and pTG6558 are digested with *Eco*RV, a unique site located between the two expression cassettes (pac gene and E1 region). A 1.88-kb *Eco*RV-*Pvu*II fragment isolated from pBR322 (Bolivar et al., *supra*) is cloned into this site in order to increase the distance between the two promoters. pTG6564 and pTG6565, respectively, are generated.

The vectors pTG6557, pTG6558, pTG6559, pTG6564 and pTG6565 are transfected into cell line A549. As before, puromycin-resistant clones are selected and the expression of the E1 region is verified. The clones expressing E1 are intended for amplifying and propagating adenoviruses which are defective for the E1 function. The production of E1 expression products is accompanied by a cytotoxic effect, but Southern analysis does not enable vector rearrangements to be demonstrated. After infection with Ad-RSV-βgal, several clones are capable of amplifying the virus by a factor of more than 100.

3. *Construction of a complementation cell which is inducible by the Saccharomyces cerevisiae Gal4 protein*

These vectors comprise, as before, the portion of the Ad5 E1 region ranging from nucleotide 505 to 4034. However, the expression of the sequences of the E1A region is placed under the control of an inducible promoter consisting, on the one hand of the Ad2 MLP minimal promoter (TATA box and transcription initiation

signal; nucleotides -34 to +33), and on the other hand of an activating sequence of the Gal10 gene which can be activated by the Gal4 protein. The consensus activating sequence of 17 nucleotides (17MX) which corresponds to the Gal4 binding site is specified in Webster et al. (1988, Cell, *52*, 169). The transcription termination signal of the rabbit β-globin gene is placed at the 3' end of the E1B transcription unit.

A first DNA fragment comprising a dimer of the 17MX sequence (SEQ ID NO: 32 and 33) followed by the Ad2 MLP minimal promoter, and equipped at its 5' end with a *Sal*I site and at its 3' end with a *Bam*HI site, is synthesized. The *Sal*I site is rendered blunt by treatment with Klenow DNA polymerase. Separately, a second DNA fragment comprising a pentamer of the sequence followed by the same promoter, and equipped at the 5' and 3' ends with *Xba*I and *Bam*HI sites, is synthesized. After *Xba*I digestion, the end is rendered blunt by treatment with Klenow polymerase.

Each of these fragment ~~[sric]~~ is introduced into the *Bgl*II site of p poly II to generate pTG1656 and pTG1657, respectively. The following two fragments are then introduced into each of the vectors previously digested with *Pst*I-*Bam*HI: the *Pst*I-*Xba*I fragment isolated from pTG6552 (Example 6.2), and the *Xba*I-*Bam*HI fragment isolated from pTG6559 (Example 6.2). pTG1660 and pTG1661, respectively, are obtained (Figure 5).

A549 cells are cotransfected with pTG1643 (vector for the expression of the pac gene) and either pTG1660 or pTG1661. Clones are selected for their puromycin resistance and studied as described above. Approximately 50% of the A549-1660 and A549-1661 clones produce expression products of the E1 region. However, the production is accompanied by a cytotoxic effect, modifying the morphological appearance of the cells.

The integration and non-rearrangement of the plasmids in the cell genome is verified by Southern analysis. No substantial modification of the integrated plasmids (pTG1643, pTG1660 and pTG1661) can be

demonstrated in the producing clones analyzed. The inducibility of the expression of the sequences encoded by the E1A region in the presence of Gal4 can also be verified (by transformation with a plasmid permitting constitutive expression of the Gal4 protein).

After the infection of several producing clones with Ad-RSV-Bgal ~~[sic]~~ at a moi of approximately 2, two A549-1660 clones are capable of amplifying the viral stock by a factor of more than 100.

EXAMPLE 7: Formation of a complementation line for all of the functions essential to the replication of an adenovirus

A vector is constructed comprising the whole of the Ad5 adenoviral genome with the exception of the 5' ITR, the 3' ITR and the encapsidation region.

The vector pTG6528 (Example 6.1) is digested with the enzymes *Pst*I and *Bgl*II, between which there is inserted a DNA fragment ~~[sic]~~, synthesized chemically according to the standard protocol, consisting of the oligonucleotides of the OTG5039 and OTG5040 (SEQ ID NO: 34 and 35). The oligonucleotide sequence is designed so as not to re-form the *Pst*I cloning site and to introduce an *Eco*RV site. pTG1639 is obtained, which is linearized by *Eco*RV digestion and ligated to an *Xba*I-*Bam*HI fragment whose ends are rendered blunt by treatment with Klenow DNA polymerase. This fragment carries the SV40 virus transcription termination signal. Any plasmid containing a signal surrounded by appropriate restriction sites may be used in this step.

The vector pTG1640 thus generated is digested with *Bam*HI and *Bgl*II, and the fragment carrying the cassette for the expression of the pac gene is introduced into the *Bgl*II site of the vector p PolyII-Sfi/Not-14*. pTG1641 is obtained. The latter is linearized with *Not*I and treated with Klenow DNA polymerase. The 0.276-kb *Bam*HI-*Sal*I fragment isolated from pBR322 (Bolivar et al., *supra*) also treated with Klenow DNA polymerase is

introduced. This gives rise to pTG1643.

pTG1643 is linearized with *Xho*I, and an *Xho*I hybrid fragment containing a 17MX dimer followed by the TK-HSV-1 gene minimum promoter (nucleotides 303 to 450 of the sequence disclosed in the ~~Genebank~~ GenBank data bank under the reference V00467 and supplemented at the 3' end with a *Xho*I site) is inserted into this site. pTG1647 is obtained, in which the 2x17MX-TK-HSV-1 hybrid promoter is inserted in the same orientation as the cassette for the expression of the pac gene.

This construction, pTG1647, is used as a parent vector for introducing, between the *Pst*I and *Bam*HI sites, a fragment of the Ad5 genome ranging from nucleotide 505 to nucleotide 35826. In a first stage, pTG1647 is digested with *Pst*I and *Bam*HI and then ligated, on the one hand to the *Pst*I-*Cla*I fragment of pTG6552 (Example 6.2) containing the portion of the Ad5 genome from nucleotides 505 to 918, and on the other hand to the *Cla*I-*Bam*HI fragment (positions 918 to 21562) prepared from Ad5 genomic DNA. The vector thereby obtained contains the 5' portion of Ad5 with the exception of the 5'ITR and the encapsidation region.

Separately, the 3' portion of the Ad5 genome is assembled in the vector p polyII-Sfi/Not-14*. The latter is linearized with *Bam*HI, and the *Bam*HI-*Avr*II fragment (nucleotides 21562 to 28752) of the Ad5 genome and a PCR fragment corresponding to nucleotides 35463 to 35826 of Ad5 are introduced. The latter fragment is generated from Ad5 genomic DNA and using the primers OTG5024 (SEQ ID NO: 36) and OTG5025 (SEQ ID NO: 37), and contains at the 5' end a *Bam*HI site. The vector obtained is digested with *Avr*II, and the *Avr*II fragment isolated from Ad5 genomic DNA and extending from positions 28753 to 35462 is inserted.

The *Bam*HI fragment containing the adenoviral sequences is introduced into the *Bam*HI site of the vector of the preceding step containing the 5' portion of the adenoviral genome lacking the 5' ITR and the encapsidation region.

A complementation line capable of complementing all of the functions of a defective adenovirus is generated by transfection into a cell line, for instance A549, according to the protocol described in the preceding examples.

It is also possible to proceed by constructing four vectors containing virtually the whole of the adenoviral genome, which will be reassembled on a single vector in the final step.

- pTG1665 corresponds to the cloning of the *Bsp*EI fragment (nucleotides 826 to 7269) isolated from an Ad5 genomic DNA preparation into the *Xma*I site of p polyII-Sfi/Not-14*;
- pTG1664 is generated by inserting the *Not*I fragment (nucleotides 6503 to 1504) isolated from an Ad5 genomic DNA preparation into the *Not*I site of the same vector;
- pTG1662 is obtained by introducing the *Aat*II fragment (nucleotides 10754 to 23970) isolated from an Ad5 genomic DNA preparation into the *Aat*II site of p polyII.
- pTG1659 containing the 3' portion of the Ad5 genome (Example 2.3).

A fragment containing an inducible expression system, for instance the promoter described in Example 6.3 or 7 which is inducible by Gal4, or a promoter of the prior art such as the metallothionein or tetracycline promoter, is then introduced. Such a fragment is placed upstream of the 5' sequences of Ad5 (nucleotides 505 to 918) in the vector pTG1665 digested with *Aat*II and *Cla*I. Lastly, the *Not*I fragment of pTG1664, the *Aat*II fragment of pTG1662 and lastly the *Bam*HI fragment of pTG1659 are cloned successively into the above vector and at the corresponding sites.

A complementation line is generated by cotransfection of the above vector and pTG1643, and the puromycin-resistant clones are isolated. This line is intended more especially for amplifying and encapsidating the adenoviral vectors of Example 5, which are defective

for the E1, E2 and E4 functions and the late functions.

EXAMPLE 8: Formation of a complementation line for the E1 and E4 functions

The vector pTG1647 (Example 7) is digested with the enzymes *Pst*I-*Bam*HI, and 3 fragments are introduced into the vector thus treated:

- the *Pst*I-*Xba*I fragment of pTG6552 (Example 6.2) carrying the Ad5 sequences from nucleotide 505 to nucleotide 1339,
- the *Xba*I-*Sph*I fragment of pTG6552 carrying the Ad5 sequences from nucleotide 1340 to nucleotide 3665, and
- the *Sph*I-*Bam*HI fragment of pTG6554 (Example 6.2) carrying the Ad5 sequences from nucleotide 3665 to 4034 and a transcription termination signal.

The vector thereby obtained is cut with *Bam*HI, and the following three fragments are introduced into this site:

- a fragment digested with *Bam*HI-*Afl*II, generated by PCR, corresponding to the Ad5 sequence located between positions 32800 and 33104. The procedure used employs Ad5 genomic DNA as template and the primers OTG5078 (SEQ ID NO: 38) and OTG5079 (SEQ ID NO: 39),
- the *Afl*II-*Avr*II fragment isolated from Ad5 genomic DNA (nucleotides 33105 to 35463),
- the *Avr*II-*Bam*HI fragment generated by PCR using the primers OTG5024 and OTG5025 (see Example 7).

The vector thereby generated is introduced into a cell line according to the protocol described above, to form a complementation line for the E1 and E4 functions.

Moreover, such a line may also be obtained according to the following protocol:

The E4 region of the Ad5 genome (nucleotides 32800 to 35826) is re-formed in several steps. The portion ranging from nucleotides 33116 to 32800 is synthesized by PCR from Ad5 genomic DNA with the primer

pair OTG5078 and OTG5079 (SEQ ID NO: 38 and 39), and then inserted into the *Eco*RV site of M13TG130, to generate M13TG1645.

The *Bam*HI-*Afl*II fragment of the latter is subjected to a ligation reaction with the *Afl*II-*Avr*II fragment of Ad5 (nucleotides 33104 to 35463) and the vector pTG7457 digested with *Bam*HI and *Avr*II. pTG1650 is obtained.

The E4 region is then completed by obtaining the fragment corresponding to nucleotides 35826 to 35457 by PCR from an Ad5 genomic DNA preparation and using the primers OTG5024 and OTG5025 (SEQ ID NO: 36 and 37). This fragment is inserted into the *Sma*I site of M13mp18 to give M13TG1646. The *Avr*II-*Eco*RI fragment is isolated from the latter and cloned between the *Avr*II and *Eco*RI sites of pTG1650. pTG1652 is obtained.

The *Bam*HI fragment containing the E4 region of Ad5 is isolated from pTG1652 and cloned into the *Bam*HI site of pTG1643 and pTG6559 (Example 6.2) or into the *Ssp*I site of pTG6564 (Example 6.2), after the sites have been rendered blunt to generate pTG1653, pTG1654 and pTG1655 (Figure 6), respectively.

A complementation cell capable of complementing *in trans* E1 and E4 functions is generated by conventional techniques, by:

(1) transformation of pTG1653 into cell line 293, or

(2) transformation of pTG1654 or pTG1655 into cell line A549.

Generally speaking, the expression of the products of the E1 and E4 regions is accompanied by a cytotoxic effect. A number of 293-1653 clones are capable of complementing both adenoviruses from which E1 has been deleted and adenoviruses from which E4 has been deleted.

Another alternative consists in proceeding as follows.

The vector M13TG1646 is subjected to a directed mutagenesis with the mutagenic oligonucleotide OTG5991 (SEQ ID NO: 40), with the object of deleting the promoter of the E4 region and inserting an *Hpa*I site. The mutated

vector is designated M13TG6522. It is digested with *Pst*I, treated with phage T4 DNA polymerase and then with *Avr*II and ligated with a purified *Eco*RI (Klenow)-*Avr*II fragment of pTG1652 (Example 8), to give pTG6595. The latter is cleaved with *Hpa*I, and the 0.8-kb fragment obtained from pTG5913 (Figure 7) after *Bgl*II and *Bam*HI digestion and Klenow treatment is introduced. pTG6596 is generated, in which the E4 region (positions 32800 to 35826) is placed under the control of the TK promoter. For guidance, pTG5913 carries the TK-HSV-1 gene, and the *Bgl*II-*Bam*HI fragment corresponds to the promoter of this gene (Wagner et al., 1981 Proc. Natl. Acad. Sci., USA, *78*, 1441-1445).

In parallel, the vectors pTG1643 and pTG6559 (Example 6) are linearized with *Bam*HI, and a synthetic fragment originating from the recombination of the oligonucleotides OTG6141 and OTG6142 (SEQ ID NO: 41 and 42) is inserted, to obtain pTG8508 and pTG8507, respectively.

These latter are cleaved with *Bam*HI before the purified *Bam*HI fragment of pTG6596 containing the cassette for the expression of E4 is introduced. The vectors pTG8512 (Figure 8) and pTG8513 (Figure 9) are generated.

Moreover, introduction of the *Bam*HI fragment of pTG1652 into the vector pTG8508 or pTG8507 linearized with the same enzyme leads to pTG8514 and pTG8515, respectively (Figures 10 and 11).

Cell lines transfected with pTG8512 or pTG8515 will enable an adenovirus which is defective for the E4 function to be complemented, whereas those resulting from pTG8513 or pTG8514 transfection are intended for amplifying and propagating adenoviruses which are defective for the E1 and E4 functions. Similarly, the transfection of pTG8512 or pTG8515 into 293 cells will enable adenoviruses which are defective for E1 and E4 to be complemented.